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Dr. Seth Willey
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Dear Seth:

Thank you for allowing me to participate in the review process of this important and complex issue. I have completed the review of the report entitled "Comprehensive Analysis of Molecular Phylogeographic Structure Among Meadow Jumping Mice (*Zapus hudsonius*) Reveals Evolutionary Distinct Subspecies" by King *et al*. I have considered all of the materials that you provided in order to make my evaluation.
Attached please find my comments and an updated version of my CV.
I hope that you find them useful and please let me know if have any questions or if you need any additional information.

Sincerely,

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1) Please analyze the techniques used for the population and the phylogenetic evaluation of *Zapus hudsonius preblei* and other taxa. Were the appropriate methodologies and markers used?

Yes, I think that this work shows very clearly how we need adequate data and use of appropriate lab techniques before we can draw conclusions on such an important issue as the phylogenetic distinctiveness of an endangered subspecies. In this study, the authors clearly and methodically demonstrate how by using an improved sampling strategy, adequate types of samples, more mtDNA sequence data (a fragment of the control region and the entire cytochrome b protein coding gene), and nuclear DNA microsatellite data (21 microsatellite markers!) one can more confidently and reliably draw conclusions on the phylogeographic structure of five subspecies of *Zapus hudsonius* and particularly on the evolutionary distinctiveness of *Z. h. preblei* from neighboring subspecies. In addition, all of the methodologies for data analysis for both mtDNA and microsatellite data are appropriate and up to date. These techniques are commonly used to answer questions related to phylogenetic distinction, genetic differentiation and population structure analysis.

2) Based on the data presented in the report, do you support the authors' conclusions about the taxonomic validity of *Z. h. preblei* and neighboring subspecies?

Yes, the authors present compelling evidence that suggests that *Z. h. preblei* should be considered a distinct evolutionary lineage from *Z. h. intermedius* and *campestris*. The magnitude of differentiation that they report for the five subspecies that were selected for the analysis is highly significant for almost all of the tests and comparisons that they executed. Remarkably, and contrary to what was found in the Ramey et al. (2005) study, they found unique sets of haplotypes for each subspecies (for both cyt b and CR) and significant allele frequency differences (based on the 21 microsatellite loci examined) between all of the 5 subspecies which was also corroborated by the high percentage of correct assignment for *Z. h. preblei, campestris, intermedius, pallidus* and *luteus*.

3) **Based on the data presented in the report, do you support the authors' conclusions that Z. *h. preblei* is comprised of at least two population segments worthy of individual management consideration?**

Yes, their refined microsatellite analysis using *Structure* clearly suggests that there are six distinct clusters among the four subspecies with *Z. h. preblei* divided into a North and South cluster. In addition, no mtDNA cyt b haplotypes were shared between the North and South groupings and only 1 of the 4 control region haplotypes was shared between them. In my opinion these results suggest that separate management consideration is merited.

4) Are there possible alternative interpretations of the data that could be drawn from the genetics data? How likely are these possibilities?

No, I think that they did a great job defending the interpretation of their data and refuting Ramey et al. (2005). I agree with their contention that Ramey et al.'s critical test of uniqueness for *Z. h. preblei* stating that "greater molecular variance be demonstrated between subspecies than within" may be too stringent a criterion for determining subspecific uniqueness.

5) What additional analysis, if any, is needed to verify the study's assertions and why?

I agree with King et al.'s assertions that a more detailed comparison similar to this study but for all 12 of the subspecies of *Z. hudsonius* is really needed to get an overall picture of differentiation of all the subspecies in this group and is needed anyway for an adequate revision of the group surveying for genetic variation throughout the entire species range. How can we justify revising the taxonomy of a group when we do not have all of the members of that species represented?

In addition, although not necessary to verify the study's assertion, I think it would have been a more interesting study had they incorporated coalescence analyses into their report, as I think it can contribute information about history regardless of whether haplotype phylogenies are structured or not. It is well documented that a combination of phylogenetic analysis of haplotypes and coalescence analyses provides powerful tools for investigating recent population history. In my opinion, it could be just as important to learn that a group's history is one of population and range expansion as it is to document their history of isolation.

6) The conclusions of Ramey et al. (2005) and King et al. (2006) would appear to conflict. What are the most likely explanations for this conflict?

These two studies have very different conclusions about the evolutionary distinctiveness of *Z. h. preblei*. The difference in the conclusions is not just a difference in how they interpreted the results of their data but is due to basic differences in the methods that the two studies employed. The King et al. study had a superior sampling design that increased the likelihood of detecting more of the genetic variation, a better more reliable source of the tissue sample for DNA extraction, a greater amount of mtDNA sequence data, and a much higher number of nuclear microsatellite loci that provided for more robust estimates of population genetic parameters and an increased resolution of the genetic structure in *Z. hudsonius*. The King et al. study also differed in their application of the statistics used in their test of molecular variance. They correctly use Φ_{st} statistic rather than F_{st} for the estimate of variation between subspecies. Finally, the two studies had different criteria to determine evolutionary uniqueness.

7) Has this new information changed your conclusions regarding the synonymizing of *Z. h. preblei* and *Z. h. campestris* and neighboring subspecies? Please elaborate as necessary.

After reviewing the first report by Ramey et al. I was not fully convinced that they had gathered enough information to support their conclusions. I stated, *"While I support the taxonomic interpretations of the authors based on the data they presented, I would strongly suggest that they consider analyzing microsatellite data to corroborate their findings." "I would strongly recommend that they add microsatellites to their study. This is an important and worthwhile conservation genetics issue to pursue and will perhaps set precedence as to how we determine the management of an endangered species with a controversial taxonomy. We need at least this additional evidence and while I agree that microsatellite development is expensive and time consuming, I am aware that there are microsatellite primers already published for Zapus and that they may be useful to look at finer scale patterns of gene flow and population structure. (See S. N. Vignieri. 2003. Isolation and characterization of eight highly variable microsatellite markers in the Pacific jumping mouse (Zapus trinotatus). Molecular Ecology Notes 3:638-640.)***". The fact that they had recovered haplotypes in *Z. h. preblei* from *Z. h. campestris* seemed to suggest that there was recent gene flow between the two and if confirmed it would be an important factor to take into consideration, but I had reservations about their conclusions. In my previous review I also stated " *In the ancient DNA literature, the need for confirmation of*

sequences in other labs to rule out contamination is not uncommon and the authors (if they have not already done so) may consider testing the few individuals that appear in the Z. h. campestris that appear in the Z. h. preblei clade in an independent lab".

In my opinion, the King et al. study has objectively and methodically addressed the issues, and based on the scientific evidence that they presented in this study, I disagree with the synonymizing of *Z. h. preblei* and *Z. h. campestris* and neighboring subspecies. Finally, while I agree with King et al. that fresh, modern samples are a more reliable source of DNA than ancient DNA analysis from museum specimens, I think that we should not use this study to under appreciate ancient DNA studies conducted under strict laboratory conditions and with the appropriate replicated controls, as they have proven to be an invaluable tool for studying difficult to obtain or extinct species and are a powerful technique that allows us to look at past levels of genetic variation.